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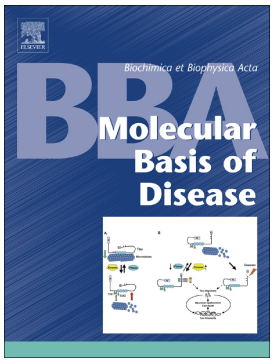

Low galactosylation of IgG associates with higher risk for future diagnosis of rheumatoid arthritis during 10 years of follow-up

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Low galactosylation of IgG associates with higher risk for future diagnosis of rheumatoid arthritis during 10 years of follow-up

Running title: IgG N-glycans in rheumatoid arthritis

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Abstract

Antibodies are known to have an important role in the development of rheumatoid arthritis (RA), one of the most prevalent chronic inflammatory diseases which primarily involves the joints. Most RA patients develop autoantibodies against immunoglobulin G (IgG) and changes in IgG glycosylation have been associated with RA. We undertook this study to determine whether altered IgG glycosylation precedes the disease diagnosis. We studied IgG glycosylation in RA in two prospective cohorts (N=14,749) by measuring 28 IgG glycan traits in 179 subjects who developed RA within 10-years follow-up and 358 matched controls.Ultra performance liquid chromatography method based on hydrophilic interactions (HILIC-UPLC) was used to analyse IgG glycans. Future RA diagnosis associated with traits related to lower galactosylation and sialylation of IgG when comparing the cases to the matched controls. In RA cases, these traits did not correlate with the time between being recruited to the study and being diagnosed with RA (median time 4.31 years). The difference in IgG glycosylation was relatively stable and present years before diagnosis. This indicates that long-acting factors affecting IgG glycome composition are among the underlying mechanisms of RA and that decreased galactosylation is a pre-existing risk factor involved in the disease development.

Keywords: rheumatoid arthritis, immunoglobulin G, N-glycans, biomarker, risk factor

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease which primarily involves joints and has an incidence of 0.5 – 1%[1], making it one of the most prevalent chronic inflammatory diseases[2]. Even though mechanisms of the disease development are yet to be completely identified, antibodies are known to be important players in that processand connected with the disease severity[2–4].Autoantibodies against citrullinated peptides (ACPAs) and immunoglobulin G (IgG) are particularly important in the diagnostics of RA. An early diagnosis is central to the treatment of RA, as starting a therapy as soon as possible can prevent or halt joint damage in many patients[5].

IgG is the most abundant immunoglobulin in human circulation and a majority of RA patients produce autoantibodies binding to IgG, collectively denominated rheumatoid factor (RF). IgGitself is a glycoprotein and both its antigen binding region (Fab) as well as the fragment crystallizableregion(Fc) are known to be N-glycosylated[6]. These glycans are mostly biantennary complex-type N-glycans carryingvarying levels of a core fucose, *N*-acetylglucosamine (GlcNAc), galactose and sialic acid[7]. Glycosylation of IgG has a major effect on its effector and antigen binding functions and may also affect its role in RA.

IgG glycosylation changes are known to be associated with RA since 1985 when Parekh et al. showed that galactose and sialic acid levels were lower in patients with RA[8]. Moreover, these changes follow the disease remission during pregnancy[9–11]and an efficient therapy treatment[12–15] as well as relapses and progression of the disease[16]. Besides RA, N-glycosylation is shown to be altered in other autoimmune diseases such as systemic lupus erythematosus[17] and inflammatory bowel disease[18] as well as in inflammatory diseases

without autoimmune character[19–21].Some authors have also suggested that there is a "sugar print" characteristic for RA which enables distinguishing it from different similar diseases[22,23]. Whether changes in IgG glycosylation contribute to RA development is unclear, although a recent Mendelian randomization study does not support a simple causal relationship[24].

Recent development of new analytical methods and approaches has enabled the analysis of IgG N-glycome in a larger number of patients and with higher accuracy[25,26]. Ultra performance liquid chromatography based on hydrophilic interactions (HILIC-UPLC) analysis of 2-aminobenzamide (2-AB) labelled PNGase F released N-glycans has been proven to be reproducible and robust[25,27]. We used this method to analyse IgG glycosylation profiles of 179individuals that were diagnosed with RA during 10-year follow-up after sampling (cases), and 358 matching controls that did not develop RAin the same period. Herein we show that the IgG glycan aberrancy can be detected years before the onset of the disease and considered as a novel risk factor for RA.

2. Materials and Methods

2.1.Study population.

RA cases and controls were originally recruited to the FINRISK 2002 (N=8,595) and 2007 (N=6,154) cohorts. FINRISK cohorts are population-based samples of Finns, described in more detail previously[28]. During the follow-up, 179 incident RA cases were identified among the study participants using the Finnish hospital discharge and causes of death registers with ICD-codes: M05-M13, M32, M33, M45 (ICD-10) / 710, 714, 420, 725 (ICD-9) / 712, 734 (ICD-8) and the social insurance institution of Finland (KELA) special reimbursement for RA medication (KELA code 202). Two controls per case were frequency matched by gender, 5-year age group, FINRISK year and study region. Individuals who developed other autoimmune or chronic diseases were excluded from the present study. The median follow-up time was 4.31 years for cases (range 0.025 to 9.83 years) and 9.80 years for controls (range 0.33 to 9.94 years).

2.2.IgG isolation, glycan release and labelling.

The whole procedure was performed as previously reported[25]. Briefly, IgGs were isolated using Protein G 96-well plate (BIA Separations, Slovenia) from the serum samples. The isolated IgGs were denaturated with the addition of sodium dodecyl sulphate (SDS) (Invitrogen, USA) and by incubation at 65°C. The excess of SDS was neutralized with Igepal-CA630 (Sigma-Aldrich, USA) and N-glycans were released following the addition of PNGase F (Promega, USA) in Phosphate Buffered Saline (PBS). The released N-glycans were labelled with 2-AB. Free label and reducing agentwere removed from the samples using hydrophilic interaction liquid chromatography solid-phase extraction (HILIC-SPE). Glycans were eluted with ultrapure water and stored at –20°C until use.

2.3.*Ultra-performance liquid chromatography*.

Fluorescently labelled N-glycans were separated by HILIC on an Acquity UPLC instrument (Waters, USA) consisting of a quaternary solvent manager, sample manager, and an FLR fluorescence detector set with excitation and emission wavelengths of 250 and 428 nm, respectively. The instrument was under the control of Empower 3 software, build 3471 (Waters). Labelled N-glycans were separated on a Waters BEH Glycan chromatography column, 100 × 2.1 mm i.d., 1.7 µm BEH particles, with 100 mM ammonium formate, pH 4.4, as solvent A and ACN as solvent B. The separation method used a linear gradient of 25-38% solvent A at flow rate of 0.40 ml/min in a 27 min analytical run. Samples were maintained at 10°C before injection, and the separation temperature was 60°C. Data processing was performed using an automatic processing method with a traditional integration algorithm, after which each chromatogram was manually corrected to maintain the same intervals of integration for all the samples. The chromatograms were all separated in the same manner into 24 peaks as previously reported[7]. The amount of glycans in each peak was expressed as % of total integrated area. Derived traits were calculated according the following formulas: for agalactosylated G0 = GP1+GP2+GP3+GP4+GP6, with one galactose G1 = GP7+GP8+GP9+GP10+GP11, with two galactoses G2 = GP12+GP13+GP14+GP15 and sialylated glycans S = GP16+GP17+GP18+GP19+GP21+GP22+GP23+GP24.

2.4.*Data analysis.*

The glycan variables were first inverse normal transformed and standardized to mean=0 and standard deviation=1. Then, for each of the transformed glycan variables, a linear regression model adjusted for sex, age, age group ([,30 y}, [30 y, 40 y}, [40 y, 55 y}, [55 y, 70 y}, [70 y, }),

and an age*sex interaction term was fitted and the residuals from the model were extracted. The association of the residuals with RA was then tested with incident disease (yes/no)using logistic regression and, in cases only, with time from sampling to diagnosis (years) using linear regression. To take into account multiple testing in the presence of substantial correlation between the glycan variables, principal component analysis was used. As the first 19 principal components explained more than 99% of the variance in the glycan residuals, the P-values for the logistic regression tests were adjusted by multiplying the nominal P-values by 19. P-values for tests with time to diagnosis as the dependent variable are presented without adjusting for multiple testing.

3. Results

IgG N-glycans were released by PNGase F, labelled with 2-AB andanalysed by HILIC-UPLC. Obtained chromatograms were integrated into 24 peaks which mostly contained individual N-glycans (Figure 1)[7]. The association of the N-glycanswith incident RA was tested using logistic regression (Table 2).

Glycan peaks GP3, GP4 and GP6were increased in RA cases when compared to controls (nominal P-value<0.05).These peakscontain agalactosylated glycan structures A2B, FA2 and FA2B, respectively. On the contrary, the proportion of total area comprised by the glycan peaks containing structures with one galactose (GP8 and GP9) or two galactoses (GP12, GP13, and GP14) was smaller in cases, as were the % areas of the peaks GP18 and GP23 containing sialylated IgG glycans (nominal P-value<0.05). The derived variables for agalactosylation (G0), monogalactosylation (G1), and digalactosylation (G2) were also nominally significant. The associations of the peak GP6 (agalactosylated glycan FA2B), total agalactosylation (G0), and total monogalactosylation (G1) with RA remained statistically significant after adjusting the P-values for multiple testing (P_{GP6}=0.008, P_{G0}=0.027, P_{G1}=0.029, Table 2).

The association oflow IgGgalactosylation with future diagnosis of RA may reflect changes occurring during early phase of the disease, or, alternatively, it may indicate that low IgG galactosylation is a pre-determined or acquired risk-factor. Aiming to distinguish between these two alternative hypotheses, we checked whether the observed changes associate with the time between sampling and RA diagnosis (Table 2, Figure 2).

The only variable showing a nominally significant association was GP9 that correlated with a lower risk of RA and a longer time between taking the blood sample and diagnosis with

RA, but neither of these associations remained statistically significant after adjusting the P-value for multiple testing. Noneof the N-glycan variables that robustly associated with RA risk (GP6, G0, and G1) correlated with the time between RA diagnosis and the date of participating in the study (Table 2, Figure 2).

4. Discussion

Results of our study are in accordance with the previous studies that reported decreased galactosylation in RA [8,16,29]. The decreasein galactosylation and sialylationin RAhas been known for a long time[8] and was observed in multiple independent studies[16,29]. The decrease inIgG galactosylation has beenregularly associated with RA[8,10,15,30–36], while changes in sialylation were reported to be independent of galactosylation[9]and the decrease in sialylation wasfar less reported[8,10,15,37]. It is known that both anti-inflammatory and pro-inflammatory activities of IgGare determined by which Fcγ receptor they preferentially bind toand that the receptor affinity depends on the composition of the glycan linked to the 297 asparagine. IgG carrying G0 glycans hasincreased affinity to FcγRIII, an activating FcR, and the serum lectinmannose-binding protein[38,39]. Moreover, besides the classical and lectin pathways of complement activation, IgG bearing G0 is able to activate an alternative complement pathway[40].Such G0-bearing IgGsare thus uniquely capable of setting autoimmunity.To the contrary, higher sialylation of IgG decreases its affinity for FcγRIIIand increases the expression of FcγRIIB,an inhibitory FcR[38].

This is not the first study which reported that IgG glycosylation changes could be observed before the onset of symptoms[31,41,42], yet there is disagreement between studies about the time point when changes are starting to be visible. Ercan et al. concluded that the changes could be noticed at least 3.5 years[41] and Young at al. observed them two years prior the onset of symptoms[31], while Rombouts et al. stated that a decrease in galactose residues occurred around three months prior to diagnosis and only in ACPA-IgG[42]. In this study we

detected robust association of low IgG galactosylation (andnominally significant association of low IgG sialylation)and risk for RA diagnosis during 10 years of follow-up.In our data, the median time from donating the blood sample used for glycan measurement to the subsequent diagnosis with RA was 4.31 years, ranging from less than two weeks to nearly ten years. Despite the wide time range we did not observe any statistically significant correlation between IgG glycosylation and the time from sampling to diagnosis, indicating that the decreased galactosylation is a pre-existing risk factor involved in the disease development.

Low IgG galactosylation might be genetically pre-determined, or it might be acquired through the action of environmental factors. The heritability of RA is estimated to be some 40-65% and 20% for seropositive and seronegative arthritis, respectively[2]. The heritability of IgG N-glycomecomposition is also known to be relatively high[7].Yarwood et al. recently looked for shared genetic factors between RA and IgG N-glycome using IgG N-glycosylation associated single nucleotide polymorphisms (SNPs) and found no association between them and risk for RA[24].This suggests low IgGgalactosylation may not be a genetically pre-determined risk factor. The differences in IgG N-glycans between RA cases and healthy controls precede diagnosis for years, which coincides with the preclinical period of the disease known as pre-rheumatoid arthritis (pre-RA). This period includes a period in the RA development where genetics and environmental factors interact and which is followed by development of disease-related autoantibodies and symptoms without clinical evidence of RA[43–45]. All these stages can be present years before RA establishment. Knowing that environmental risk factors such as smoking, obesity, periodontitis, hormones, diary and infections together with genetic risk factors play an important role in the first stage of pre-RA[44], and some of these factors are known to affect the IgG glycome [7,46].Therefore, this indicates that stable long-acting factors affecting

IgG glycome composition are among of the underlying mechanism of RA and makes IgG glycosylation a novel risk factor for RA which should be considered for risk estimation of RA[47]. Moreover, knowing that IgG N-glycans are affecting inflammatory activity of IgG, this may be an indication that the glycans are not only a biomarker but a functional effector of the disease..

As joint destruction increases with disease progression, new biomarkers should be found to establish the diagnosis early to reduce or prevent consequent damage[2]. Today, the diagnosis is primarily based on serological testing for RFs and ACPAs[16], and they are known to be present in serum long before the onset of the disease[48].However, the aforementioned biomarkers are neither detectable in every RA patient (merely 70%) nor are selective for RA since they could also result from other diseases[16]. Therefore, IgGglycans have a considerable potential to be a biomarker of the disease; since they have been shown to have, combined with RF titters, a positive predictive value of 94% for RA diagnosis[31]. Moreover, agalactosylatedIgG levels are known to correlate with the disease remission, progression and successful therapy treatment[16]. Indeed, IgGglycans follow a temporary improvement of RA during pregnancy by an increase in IgGgalactosylation and sialylation as well as a decrease of the same glycan traits after delivery, which correlates with a postpartum relapse[10]; those changes could be attributed to higher estrogen levels in pregnancy which is known to modulate IgGgalactosylation[49].

Even though the method applied in this study for IgG N-glycome analysis,as well as other analytical techniques which require HPLC/UPLC and mass spectrometry, are not yet routinely used in the clinic for disease diagnostic, undergalactosylatedIgG could be easily estimated by ELISA techniques using protein G in combination with

biotinylated*Psathyrellavelutina*lectin (PVL), which preferentially interacts with the *N*-acetylglucosaminegroup exposed at the termini of sugar chains in agalactosylatedIgG. Moreover, the assay was able to distinguish RA from other autoimmune diseases[23].

In conclusion, in this study we showed that IgG N-glycosylationcorrelates with RA years before RA diagnosis and indicates an active involvement of N-glycans in the disease pathology. Moreover, these early changes ofIgG N-glycome may thus be useful in constructing a sensitive and specific test for early RA diagnosis, needed to enable prompt treatment and prevent irreversible joint destruction as well as to evaluate disease progression, remission and proper treatment.

Figure legends

Figure 1. Representative chromatogram of 2-AB labelled N-linked glycans released from IgGs isolated from human serum and separated by HILIC-UPLC. The integration areas, together with a major structure presented in each glycan peak are given. Glycan peaks are numbered from GP1-GP24, as used in the paper.

Figure 2. Agalactosylation of IgG and time from sampling to diagnosis with rheumatoid arthritis in cases only. Residual G0 is independent of the time from recruitment to diagnosis in cases. The derived agalactosylation variable G0 was formed by summing the peaks GP1, GP2, GP3, GP4, and GP6. It was then inverse normal transformed and a linear regression model adjusted for cohort, age, sex, age*sex interaction term, and age group ([,30}, [30, 40}, [40, 55}, [55, 70}, [70, }) was fitted to the transformed variable and residuals from that model were extracted. The dots represent individual measurements while the box plots summarize data from individuals across the four groups of [0.0, 2.5}, [2.5, 5.0}, [5.0, 7.5}, and [7.5-10} years.

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Table 1. Age and sex distribution of the study sample.

Age group	Control men	Control women	Case men	Case women
(,30]	6 (1.7 %)	8 (2.2 %)	3 (1.7 %)	4 (2.2 %)
(30,40]	14 (3.9 %)	28 (7.8 %)	7 (3.9 %)	14 (7.8 %)
(40,55]	34 (9.5 %)	88 (24.6 %)	17 (9.5 %)	44 (24.6 %)
(55,70]	76 (21.2 %)	68 (19 %)	38 (21.2 %)	34 (19 %)
(70,]	16 (4.5 %)	20 (5.6 %)	8 (4.5 %)	10 (5.6 %)

Table 2. Association of rheumatoid arthritis and IgG N-glycome.

Glycan trait	OR (95% CI)*	P (OR)*	$P_{adjusted}$ (OR)*	Beta (95% CI)**	P (beta)**
S	0.82 (0.66 to 1)	0.064	1	-0.2 (-0.64 to 0.24)	0.38
G0	1.5 (1.2 to 1.9)	0.0014	0.027	-0.0034 (-0.5 to 0.49)	0.99
G1	0.74 (0.61 to 0.89)	0.0015	0.029	0.4 (-0.012 to 0.81)	0.059
G2	0.71 (0.56 to 0.91)	0.007	0.13	0.00033 (-0.49 to 0.49)	1
GP1	1.2 (0.98 to 1.5)	0.083	1	-0.18 (-0.63 to 0.26)	0.43
GP2	1 (0.86 to 1.3)	0.67	1	-0.094 (-0.5 to 0.31)	0.65
GP3	1.2 (1 to 1.5)	0.036	0.68	0.21 (-0.21 to 0.63)	0.32
GP4	1.4 (1.1 to 1.8)	0.0045	0.085	0.037 (-0.44 to 0.52)	0.88
GP5	1.1 (0.94 to 1.4)	0.2	1	-0.018 (-0.43 to 0.39)	0.93
GP6	1.5 (1.2 to 1.9)	0.00042	0.008	-0.17 (-0.66 to 0.32)	0.5
GP7	0.85 (0.7 to 1)	0.074	1	-0.082 (-0.46 to 0.3)	0.67
GP8	0.77 (0.63 to 0.93)	0.0063	0.12	0.35 (-0.057 to 0.76)	0.094
GP9	0.81 (0.67 to 0.97)	0.026	0.49	0.41 (0.018 to 0.8)	0.042
GP10	1.2 (0.96 to 1.4)	0.13	1	-0.22 (-0.64 to 0.2)	0.31
GP11	1.2 (0.99 to 1.5)	0.061	1	-0.15 (-0.57 to 0.27)	0.49
GP12	0.78 (0.64 to 0.95)	0.013	0.25	-0.19 (-0.61 to 0.22)	0.36
GP13	0.83 (0.68 to 1)	0.045	0.86	-0.019 (-0.41 to 0.37)	0.92
GP14	0.71 (0.56 to 0.92)	0.008	0.15	0.057 (-0.45 to 0.56)	0.83
GP15	0.92 (0.75 to 1.1)	0.42	1	-0.26 (-0.66 to 0.15)	0.22
GP16	0.99 (0.83 to 1.2)	0.93	1	0.17 (-0.22 to 0.55)	0.4

GP17	0.91 (0.75 to 1.1)	0.33	1	-0.36 (-0.77 to 0.044)	0.082
GP18	0.77 (0.61 to 0.97)	0.029	0.56	-0.19 (-0.67 to 0.29)	0.44
GP19	1 (0.83 to 1.2)	0.99	1	-0.18 (-0.56 to 0.19)	0.34
GP20	0.92 (0.77 to 1.1)	0.41	1	0.19 (-0.18 to 0.56)	0.32
GP21	0.87 (0.72 to 1)	0.14	1	-0.017 (-0.4 to 0.37)	0.93
GP22	0.96 (0.8 to 1.2)	0.69	1	-0.19 (-0.58 to 0.2)	0.35
GP23	0.79 (0.65 to 0.96)	0.019	0.37	-0.0036 (-0.41 to 0.4)	0.99
GP24	1 (0.84 to 1.2)	0.98	1	-0.26 (-0.65 to 0.12)	0.18

Glycan variables were first inverse normal transformed and a linear regression model adjusted for cohort, age, sex, age*sex interaction term, and age group ([,30}, [30, 40}, [40, 55}, [55, 70}, [70, }) was fitted for each glycan. Residuals from these models were extracted and used to test for association with rheumatoid arthritis (yes/no) using logistic regression and with time to diagnosis from sampling (cases only) using linear regression. P-values for tests with rheumatoid arthritis (yes/no) were adjusted for multiple testing by multiplying them by 19. P-values for tests with time to diagnosis from sampling as the dependent variable are presented without adjusting for multiple testing. OR - odds ratio, CI - confidence interval, GP - glycan peak, G0 – agalactosylated N-glycans, G1 – N-glycans with one galactose, G2 – N-glycans with two galactoses, S – sialylated N-glycans.

* Logistic regression for rheumatoid arthritis (yes/no)

** Linear regression for time from sampling to diagnosis with rheumatoid arthritis (years).

Highlights

- Future diagnosis of rheumatoid arthritis is associated with lower galactosylation of IgG
- IgG glycosylation alterations are present years before diagnosis
- Glycosylation is a pre-existing risk factor involved in the disease development

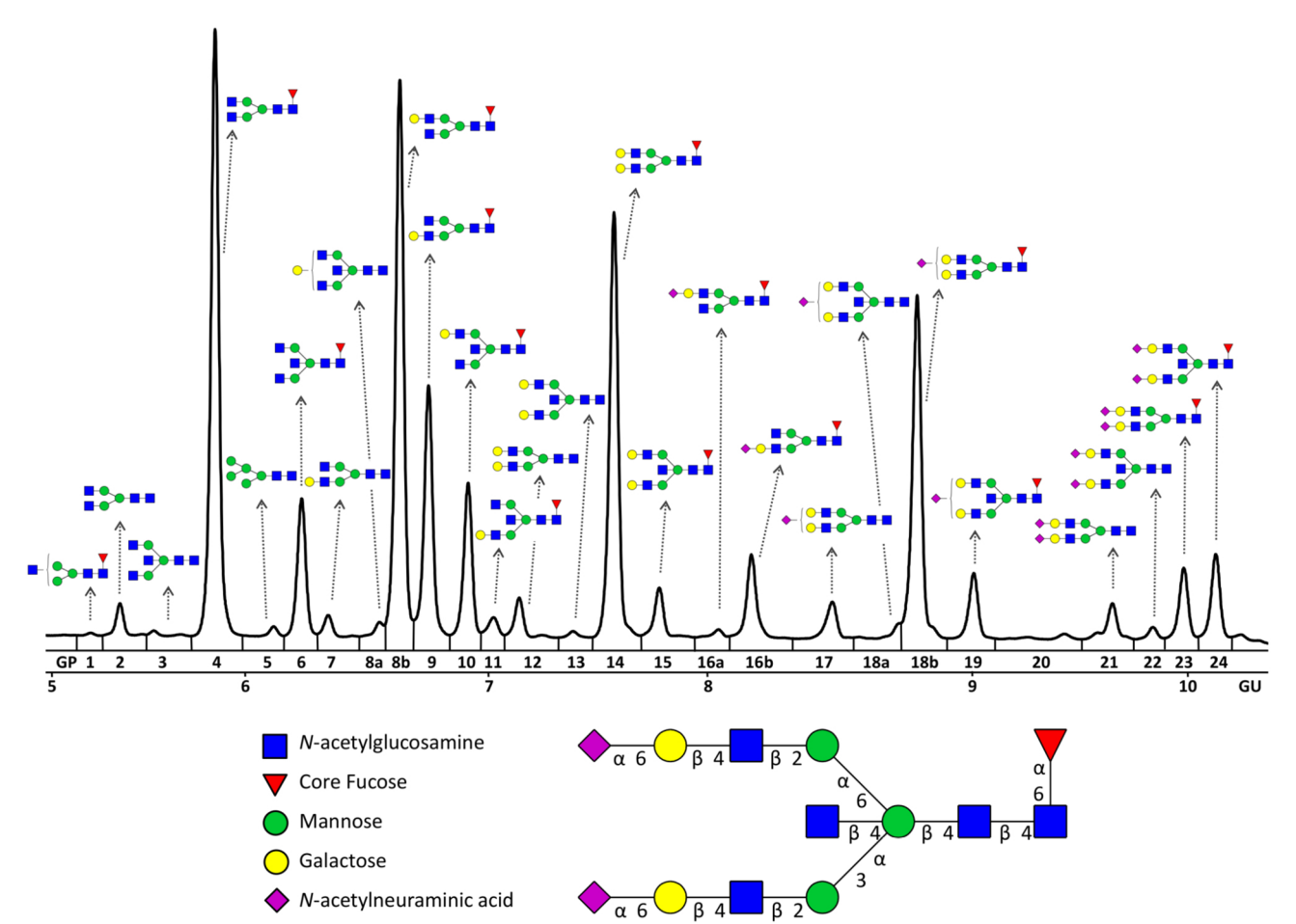
GP 1 2 3 4 5 6 7 8a 8b 9 10 11 12 13 14 15 16a 16b 17 18a 18b 19 20 21 22 23 24
5 6 7 8 9 10 GU
N-acetylglucosamine
Core Fucose
Mannose
Galactose
N-acetylneuraminic acid
α 6
β 4
β 2
α 6
β 4
β 4
β 4
α 6
α 3
α 6
β 4
β 2

Figure 1

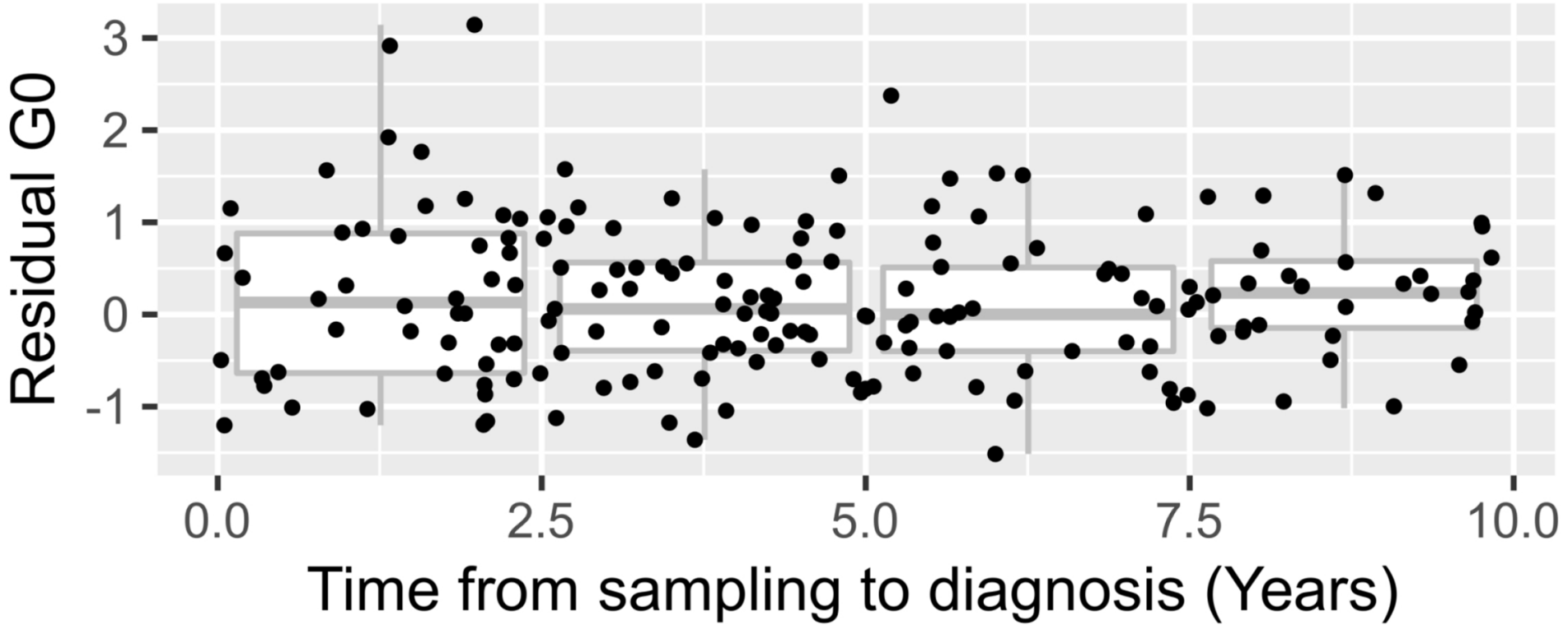
Residual G0
3
2
1
0
-1
0.0
2.5
5.0
7.5
10.0
Time from sampling to diagnosis (Years)

Figure 2